FILE 'CAPLUS, BABS, CBNB, CEN, CIN, DKILIT, IFIPAT, JICST-EPLUS, PASCAL, PLASNEWS, PROMT, RAPRA, SCISEARCH, TEXTILETECH, USPATFULL, USPAT2, WPIDS, WTEXTILES' ENTERED AT 13:28:13 ON 30 JUL 2002

L1	372062	S	STARCH
L2	43900	S	L1 AND POTATO
L3	1028	S	L2 AND CASSAVA
L4	576	S	L3 AND RICE
L5	296	S	L4 AND BARLEY
L6	171	S	L5 AND MAIZE
L7	109	S	L6 AND CORN
L8	109	S	L7 AND WHEAT
L9	1	S	L8 AND VISCOAMYLOGRAPH

L9 ANSWER 1 OF 1 USPATFULL
AN 1999:117673 USPATFULL
TI **Starch** purification by thermally tolerant broad pH range proteolytic enzymes
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Mu-Forster, Chen, Edison, NJ, United States
PA Rutgers University, Piscataway, NJ, United States (U.S. corporation)
PI US 5959102 19990928
AI US 1997-886169 19970630 (8)
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CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention is directed to purifying **starch** granules from **starch**-bearing crops, preferably **maize**, which include treating **starch** granules with a thermally tolerant, broad pH range proteolytic enzyme that is specific for surface-associated proteins. Also disclosed are purified **starch** granules which are substantially free of surface-associated proteins. Uses of the isolated **starch** granules are disclosed.
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AB The present invention is directed to purifying **starch** granules from **starch**-bearing crops, preferably **maize**, which include treating **starch** granules with a thermally tolerant, broad pH range proteolytic enzyme that is specific for surface-associated proteins. Also disclosed are purified **starch** granules which are substantially free of surface-associated proteins. Uses of the isolated **starch** granules are disclosed.
SUMM The invention is directed to improved methods for producing high quality, purified **starch** from **starch**-bearing crops. In particular, the invention is directed to the enzymatic removal of surface-associated protein contaminants from **starch** granules using thermally tolerant, broad pH range proteolytic enzymes.
SUMM **Maize** (also known as **corn** in North America), is a major source of refined **starch**, i.e., cornstarch. **Starch** is produced from **maize** and other **starch** -bearing crops by either dry milling or wet milling. It is extracted from the endosperm component of the **maize** kernel, which is composed of individual **starch** granules embedded in a proteinaceous matrix. Thus, **starch** purification requires separation of the **starch** from the protein component.
SUMM **Starch** quality is strongly associated with purity, i.e., freedom from undesirable contaminants such as proteins or lipids, and **starch** production efforts are often assessed by measurement of the protein or lipid content of purified samples (Hoseney, 1994).
SUMM Thus, an integral goal of a **starch** purification process is to produce a protein- and lipid-free product (Hoseney, 1994). The purification process allows for the disassociation of residual protein and lipid from resultant **starch** granules that can interfere with thermal and pasting properties and can impart an unpleasant taste to the **starch**. During the **starch** purification

process, protein and lipid contaminants have been known to non-covalently adsorb off-flavors and pigments, thereby limiting some applications of the **starch**. Protein levels, may be used as a crude measure of **starch** purity (Eckhoff and Tso, 1991; Steinke and Johnson, 1991; Steinke et al., 1991) and are generally determined by measuring nitrogen. . .

SUMM In order to provide highly refined **starch**, additional separation processes beyond wet or dry milling may be applied to separate the **starch** from the remaining surrounding proteins. For example, heretofore, a common means for reducing **starch** granule pigmentation and flavor has been extraction with organic solvents.

SUMM . . . products than do dry milling refining processes, but wet milling is more costly than dry milling. Wet milling involves steeping **corn** kernels in a dilute aqueous solution of sulfur dioxide under controlled conditions of time, temperature, and lactic acid concentration. These. . . to soften the kernels, inhibit growth of microorganisms, and to cleave disulfide linkages of the protein matrix in which the **starch** granules are embedded, to facilitate the release of **starch** from proteins. The steep water is then collected and concentrated in order to recover soluble components. The softened **maize** is then further processed by a series of grinding and separating operations to separate the kernel into its components, the. . .

SUMM Efforts have previously been made to apply protease enzymes to reduce the steeping time and facilitate the **starch** wet milling process. Multiple enzymes (Eckhoff and Tso, 1991; Steinke and Johnson, 1991; Steinke et al., 1991) have been reported. . .

SUMM Haring et al., in U.S. Pat. No. 5,246,718, contemplates methods for improving the flavor of **starch**, especially hydrolyzed **starch** or gelling **starch** that is essentially gluten free. However, the gelling **starch** contains a significant amount of oligopeptides. The method described in Haring et al. includes incubating the **starch** with an enzymatically active peptidase, specifically, exo-peptidases obtained from food grade bacteria, to remove bitter taste from the **starch**. While this reference might arguably teach the removal of oligopeptides, having from 3 to 30 amino acids, there is no specific teaching in this reference suggesting removal of surface-associated or localized proteins from **starch** granules. In addition, the reference is devoid of any teaching suggesting removal of zein proteins using thermally tolerant, broad pH. . .

SUMM An enzymatic treatment of **starch** is known from East German patent 139 361. Described therein is a method for treating cereal **starch** containing insoluble gluten. However, it is not always desirable to enzymatically degrade gluten, which is a product of the **maize** milling process. In addition, the conventional enzyme based process previously utilized could not be employed for prolonged periods at the elevated temperatures generally utilized for **starch** processing, due to thermal denaturation of the enzymes.

SUMM A recent study (Mu-Forster et al., 1996) demonstrates that **starch** granule-associated proteins can be divided into two classes: internalized polypeptides and surface-associated polypeptides. This study further demonstrates that internalized proteins are not accessible to proteases unless these proteins are released from the **starch** granule by gelatinization.

SUMM . . . invention reflects Applicants' observation that during the commercial wet milling process, proteins, particularly zeins, become associated with the surface of **starch** granules. These particular proteins comprise 62-74% of the protein content of **maize** endosperms (Hamaker et al., 1995; Wilson, 1987) and may serve to capture pigments and off-flavors.

SUMM Heretofore, there is no process for efficiently removing granule-associated proteins, namely those localized at the **starch** granule surface without gelatinizing the **starch** granules or degrading the gluten proteins in which the **starch** granules are embedded. The present invention provides a process for efficiently removing surface-associated proteins from the **starch** granule, thus yielding whiter **starch** having significantly less pigmentation. Consequently, the process of the present invention represents an alternative to the use of organic solvents for decolorizing **maize** starches.

SUMM An added feature of the present invention is that the process yields **starch** having a protein content from 0.13 to 0.14% compared to 0.4 to 1.0% normally found with conventional processes such as wet milling. As a result of the lower protein content, the **starch** appears whiter and is less prone to form or absorb off-flavors, which are drawbacks attending **starch** purified according to conventional processes.

SUMM The present invention reflects Applicants' endeavor to develop a process of purifying **starch** that is novel and attempts to address problems associated with conventional **starch** purification processes.

SUMM Accordingly, it is an object of the invention to provide improved and efficient methods for producing high quality **starch** from **maize**.

SUMM It is a further object of the invention to provide improved methods for removing contaminating proteins bound to **starch** granules during **starch** purification processes.

SUMM It is a further object of the invention to provide a method for removing surface-associated proteins from **starch** granules and leaving the **starch** granules intact.

SUMM It is a further object of the invention to provide a method for removing surface-associated proteins, particularly zeins from **starch** granules, thereby providing a significantly whiter and blander **starch**.

SUMM It is a further object of the invention to provide an improved method for producing high quality, purified **starch** products by incubating milled **starch** with a thermally tolerant, broad pH range proteolytic enzyme such as thermolysin.

SUMM It is a further object of the invention to provide a means to facilitate disengagement of **starch** granules from non-**starch** kernel components during steeping and post-steeping milling processes.

SUMM It is a further object of the invention to provide an improved method for producing high quality, purified **starch** having a significantly less protein and lipid content compared with commercial wet-milled **starch**.

SUMM . . . claims, the present invention is related to the surprising discovery that surface-associated proteins, primarily zein proteins, coat the surfaces of **starch** granules and are liberated during the milling process, and that these hydrophobic proteins impact **starch** pigmentation, flavor and **starch** functionality.

SUMM The present invention is further related to the surprising discovery that the selective enzymatic removal of surface-associated **starch** granule proteins at elevated but sub-gelatinization temperatures, provides for an efficient and improved method for producing high quality, purified **starch** product.

SUMM . . . discovery that a thermally tolerant, broad pH range protease such as thermolysin selectively and effectively removes the surface-associated proteins from **starch** granules at sub-gelatinization temperatures.

SUMM According to an embodiment of the invention, there is provided a method for purifying **starch** obtained from **starch**-bearing crops which include treating **starch** granules with a thermally

tolerant, broad pH range protease, preferably thermolysin, at a sub-gelatinization temperature to selectively remove surface-associated proteins from the surface of the **starch** granules. Suitable **starch**-bearing crops are well known in the art and include, but are not limited to **maize**, sorghum, **wheat**, **barley**, oats, **rice**, rye, **potato**, **cassava**, sweet **potato**, millet and banana. It is preferred that the step of treating the **starch** granules with the thermally tolerant, broad pH range protease be carried out at a sub-gelatinization temperature of from about 20.degree. C. to about 68.degree. C. The upper limit of the sub-gelatinization temperature may vary with **starch**-bearing crops other than **maize**.

SUMM According to another embodiment of the invention, there is provided a method for removing internalized proteins from **starch** granules obtained from **starch**-bearing crops which includes treating the **starch** granules with a thermally tolerant, broad pH range protease, preferably thermolysin at a gelatinization temperature sufficient to remove the internalized proteins from the **starch** granules. Suitable **starch**-bearing crops include but are not limited to **maize**, sorghum, **wheat**, **barley**, oats, **rice**, rye, **potato**, **cassava**, sweet **potato**, millet and banana. Preferably, the **starch** -bearing crop is **maize**.

SUMM According to another embodiment of the invention, there is provided a method for purifying **starch** obtained from **maize** which includes treating **starch** granules which have surface-associated proteins with a thermally tolerant, broad pH range protease, preferably thermolysin at a sub-gelatinization temperature to selectively remove the surface-associated proteins from the surface of the **starch** granules. It is preferred that the step of treating the **starch** granules with a thermally tolerant, broad pH range protease be carried out at a sub-gelatinization temperature of from about 20.degree. C. to about 68.degree. C. The treatment of the **starch** granules with a thermally tolerant, broad pH range protease is preferably conducted at a pH of about 2 to about. . .

SUMM In another preferred embodiment, the surface-associated proteins removed from the surface of the **starch** granules are zeins, having a molecular weight of about 10 to about 30 kDa as measured by SDS-PAGE.

SUMM In another preferred embodiment, the step of treating the **starch** granules with thermolysin is performed in a mixture containing calcium in a concentration of from about 0.5 mM to about. . .

SUMM Another embodiment of the invention provides purified **starch** granules obtained from a **starch**-bearing crop which have been treated with a thermally tolerant, broad pH range protease and is substantially free of surface-associated proteins otherwise found on the **starch** granule. Suitable **starch**-bearing crops include but are not limited to **maize**, sorghum, **wheat**, **barley**, oats, **rice**, rye, **potato**, **cassava**, sweet **potato**, millet and banana. Preferably the **starch**-bearing crop is **maize**. It also preferred that the surface-associated proteins are zeins. Preferably the purified **starch** granules from **starch**-bearing crops are hypoallergenic and have an improved flavor relative to **starch** not treated with a thermally tolerant, broad pH range protease. Preferably the purified **starch** granules have reduced **starch** granule pigmentation relative to **starch** granules not treated with a thermally tolerant, broad pH range protease.

SUMM Another preferred embodiment of the invention provides for purified **starch** granules from **maize** having a protein content of from about 0.13 to about 0.14% relative to a protein content of 0.4 to 1.0% for **starch** not treated with a thermally tolerable, broad pH range protease.

SUMM Another embodiment of the invention provides for a **starch** product obtained from a process which includes treating the **starch** granules obtained from **starch**-bearing crops with a thermally tolerable, broad pH range protease at a sub-gelatinization temperature to selectively remove surface-associated proteins from the surface of the **starch** granules. Preferably the **starch** product is obtained from the above-mentioned process, wherein the suitable **starch**-bearing crops include but are not limited to **maize**, sorghum, **wheat**, **barley**, oats, **rice**, rye, **potato**, **cassava**, sweet **potato**, millet and banana. Preferably the **starch** product is obtained from **maize**.

SUMM A final embodiment of the present invention provides for a method of reducing pigmentation of **starch** from **maize** which includes treating **maize** during steeping or post-steeping processes or isolated **starch** granules with thermolysin to selectively remove surface-associated proteins from the surface of said **starch** granules. Preferably the surface-associated proteins removed from the surface of said **starch** granules are zeins.

DRWD FIG. 1 Thermolysin-Catalyzed Removal of Proteins from **Starch** Granules. **Starch** granules isolated from B73 **maize** endosperm were incubated with thermolysin (2 .mu.g/mg) as described in Materials and Methods at 64.degree. C. for 30 min (lanes. . . 3 and 5) of thermolysin, as indicated. In lane 1, proteins were first extracted from an equivalent quantity of gelatinized **starch** and were then incubated with thermolysin for 30 minutes.

DRWD FIG. 2 Selective Hydrolysis of Zeins from Granule Surfaces. Wet-milled **starch** granules were incubated at 64.degree. C. or 50.degree. C. for 4 hr in the absence (-) or presence (+) of. . .

DRWD FIG. 3 Zein Content of **Starch** Granules Isolated from Amyloplasts and Homogenized Whole Endosperm. Immunoblot probed with antibodies generated against the 10-kDa .delta.-zein. Lane designations: 1. Protein extracted from 2.5 mg of **starch** isolated from purified amyloplasts of 15 DAP W64 **maize**. 2. Proteins extracted from 2.5 mg of **starch** isolated by homogenization from 15 DAP W64 whole endosperm. 3. Protein extracted from thermolysin digested **starch** from 15 DAP W64 endosperm.

DRWD FIG. 4 Time Course of Zein Hydrolysis. Wet-milled **starch** granules were incubated in the absence (lane 1) or presence (lanes 2-6) of thermolysin at 2 .mu.g mg.sup.-1 with 5 mM CaCl.sub.2. Proteins remaining associated with the **starch** granules were then extracted and analyzed by SDS-PAGE. Lane 7 contains proteins first extracted from gelatinized **starch** and were then subjected to thermolysin digestion for 30 minutes before loading onto gels. M denotes molecular mass markers.

DRWD FIG. 5 Effect of pH on Zein Hydrolysis. Wet-milled **starch** granules suspended in steeping solution were incubated with thermolysin. pH values were adjusted as indicated (lanes 1-8). Lane 9 is a control with no thermolysin added. Lane 10 contains wet-milled **starch** granules washed with water 5 times before thermolysin treatment. Proteins remaining associated with the **starch** granules were then extracted and analyzed by SDS-PAGE. M denotes molecular mass markers.

DRWD FIG. 6 Effect of Calcium on Zein Hydrolysis. Wet-milled **starch** granules were incubated with thermolysin at the Ca.sup.2+ levels indicated. Proteins remaining associated with the **starch** granules were then extracted and analyzed by SDS-PAGE. Lane 1 contains a control with no thermolysin added. M denotes molecular. . .

DRWD FIG. 7 Differential Scanning Calorimetry Thermograms. Wet-milled **starch** granules were incubated in the absence (-) or presence (+) of thermolysin, and were analyzed by DSC as described under. . .

DRWD FIG. 8 **Viscoamylograph** Viscosity Profiles of Thermolysin

Treated **Starch**. Wet-milled **starch** granules were incubated in the absence (A,C) or presence (B,D) of thermolysin at 64.degree. C. (C, D) and 50.degree. C.. . .

DRWD FIG. 10 Comparison of Zein Removal from **Maize Starch** Granules by Thermolysin and Cell Extracts of L. lactis. A. Silver stained gel. B. Immunoblot. **Starch** granules were incubated at 50.degree. C. for 4 hours with thermolysin (lane 2), or with L. lactis cell extracts containing. . .

DETD The present invention provides improved methods for the production of high quality **starch** from **maize** by the efficient, selective removal of surface-associated proteins, and particularly surface-associated proteins such as zein proteins, from the surface of **starch** granules released during milling of **maize**.

DETD Investigation of surface-associated proteins on the **starch** granules of milled **maize** has surprisingly demonstrated that surface-associated proteins, such as zein proteins, coat the surfaces of **starch** granules upon kernel disruption or homogenization, and that these hydrophobic proteins impact **starch** pigmentation. Zeins are seed storage proteins present in protein bodies in the endosperm of **maize** (Hoseney, 1994). Such storage proteins provide a source of nutrients to developing seedlings (Shotwell and Larkins, 1989).

DETD In particular, the surfaces of commercially-produced wet-milled **starch** granules contain significant deposits of zein polypeptides. Since **starch** granules produced from amyloplasts by gentle mechanical release contain markedly reduced levels of zein (FIG. 3), it is possible that. . . and amyloplast membranes are destroyed and mixing of these components occurs. Irrespective of how zeins reach the surface of the **starch** granules, zeins comprise approximately 62-74% of the total protein content of **maize** endosperms (Hamaker et al. 1995), are hydrophobic and may serve to capture pigments and off-flavors, which in turn, imparts undesirable. . .

DETD It has also been surprisingly determined that it is possible to selectively remove the surface-associated zeins from such **starch** granules by enzymatic treatment that results in starches of significantly enhanced functionality.

DETD Other surface-associated proteins present on **starch** granules of **starch**-bearing crops other than **maize**, particularly seed storage proteins that can be extracted by the method provided by the present invention include but are not limited to gliadin from **wheat**; secalin from rye; hordein from **barley**; kafirin from sorghum; avenin from oats; and oryzenin from **rice** . Support from the above becomes evident when considering that these seed storage proteins are similar in structure and amino acid. . .

DETD In addition, it is to be understood that surface-associated proteins such as zeins and those occurring in other **starch**-bearing crops are generally associated with lipids, which are also removed concomitantly with the surface-associated proteins according to the present invention.

DETD In accordance with one aspect of the present invention, wet-milled **corn starch** granules are treated with a thermally tolerant, broad pH range protease selective for zein proteins at sub-gelatinization temperatures which range. . . 68.degree. C. In particular, it has been unexpectedly discovered that treatment with a thermally tolerant, broad pH range protease enables **starch** granules to be effectively treated for removal of surface-associated proteins, such as zeins, at **starch** processing temperatures. This method is also applicable to **starch** granules obtained from other suitable **starch**-bearing crops including but not limited to sorghum, **wheat**, **barley**, oats, rye, **rice**, **potato**, **cassava**, sweet **potato**

, millet and banana. The treatment of **starch** granules with a thermally tolerant, broad pH range protease can be carried out during or after processing of **starch**-bearing crops and isolation of the granules. The various methods of processing **starch** and isolating **starch** granules from suitable **starch** -bearing crops is well-known to those skilled in the art.

DETD . . . that are particularly suitable substrates for thermolysin. As stated above, other suitable proteins including but not limited to gliadin from **wheat**; secalin from rye; hordein from **barley**; kafirin from sorghum, avenin from oats; and orzyzenin from **rice** also contain a high content of hydrophobic amino acid residues. Thus, the above exemplified proteins and other proteins found in other **starch**-bearing crops which contain suitable recognition motifs for thermolysin proteolytic activity can also be effectively removed from the surface of **starch** granules.

DETD . . . compatible with steeping conditions. The term "steeping" is well-understood by those skilled in the art. Generally, the term encompasses submerging **corn** in water containing 0.1-0.2% sulfur dioxide at about 50-55.degree. C. for 30-50 hours, to soften the kernels, inhibit growth of microorganisms, remove solubles and facilitate the release of **starch** from the protein matrix of corns (Hoseney, 1994; Eckhoff and Tso, 1991). In the early phase of the steeping process,. . . (pH 3-5), with equilibrium requiring about 15-18 hours (Biss and Cogan, 1996). However, it is known that the quality of **starch** purified from **maize** steeped at pH 5.0 is essentially identical to **starch** held at pH 3.5 (Biss and Cogan, 1996). Thermolysin, is active at a pH value of at least 2.0, and can be used under most steeping conditions. Proteins localized at the surface of **starch** granules from **maize** or other suitable **starch**-bearing crops are preferably extracted, wherein the treatment of the **starch** granules with a thermally tolerant, broad pH range protease is carried out at a pH of about 2 to about. . .

DETD . . . would generally encompass an amount that effectively removes zeins or other suitable proteins from the surface of the granule. In **maize**, this can be confirmed by determining the protein content of the resulting purified **starch** granules wherein the residual protein content is 0.13 to 0.14% relative to a protein content of 0.4% to 1.0% for **starch** not treated with a thermally tolerant, broad pH range protease.

DETD In a preferred embodiment, the purification of **starch** from **maize**, according to the present invention, is conducted by wet milling, as conventionally carried out, with the improvement comprising the digestion of **starch** granule surfaces during the final wash steps that are used to cleanse the final **starch** product. This is preferably conducted by using static washers, which lengthen the process, but have the advantage of avoiding extensive. . .

DETD An alternative embodiment contemplates promoting protein disengagement from **starch** granules during milling by genetically engineering a gene for the expression of the thermolysin enzyme into the endosperm of **maize** or other **starch**-bearing crop, either by means of a suitable vector capable of expressing active thermolysin in endosperm host cells, for localized insertion into endosperm tissues or by creating a transgenic strain of **maize** or other **starch**-bearing crop capable of expressing thermolysin in endosperm tissues. Thermolysin so expressed will remain inactive until exposed to steeping conditions, when. . .

DETD In accordance with another aspect of the present invention, **starch** granules from suitable **starch**-bearing crops are treated with a thermally tolerant, broad pH range protease, preferably thermolysin at a gelatinization temperature sufficient to remove internalized protein from the **starch** granule. Gelatinization

of **starch** granules is preferably carried out at a temperature of at least 69.degree. C. The skilled artisan will appreciate that the temperature depends on whether it is desired to make the **starch** granules porous or to completely gelatinize the granule.

DETD **Starch** granule-associated protein in **maize** can be divided into two categories: (1) internalized proteins tightly-associated with **starch** granules that become accessible to protease digestion only after **starch** is gelatinized, and (2) protease accessible proteins located at the **starch** granule surface. In the examples provided hereinbelow, **starch** granules from **maize** are proteolyzed at sub-gelatinization temperatures utilizing a thermally tolerant, broad pH range protease in order to identify and selectively remove. . .

DETD In addition, as demonstrated by the examples provided hereinbelow, removal of zeins from **starch** granule surfaces by the processes of the present invention has a significant impact upon **starch** functionality and quality. Thermolysin deproteinized **starch** is significantly whiter, thus confirming the removal of undesirable pigmentation. Further, the removal of surface-associated proteins such as zeins results in the removal of objectionable flavors from **starch** yielding a blander **starch**, which in turn, produces a **starch** having an improved flavor. This characteristic of **starch** is highly desirable especially of **starch** that is incorporated into food products. (Haring et al., U.S. Pat. No. 5,246,718, incorporated by reference herein).

DETD The term "steeping" is well-understood by those skilled in the art. Generally, the term encompasses submerging **corn** in water containing 0.1-0.2% sulfur dioxide at about 50-55.degree. C. for 30-50 hours, to soften the kernels, inhibit growth of microorganisms, remove solubles and facilitate the release of **starch** from the protein matrix of **corn** (Hoseney, 1994; Eckhoff and Tso, 1991).

DETD . . . pH range proteases which are known to those skilled in the art and which will selectively remove surface-associated proteins from **starch** granules at a sub-gelatinization temperature.

DETD The term "subgelatinization temperatures" is well-understood in the art. Generally, the term encompasses temperatures wherein **starch** granules remain intact and are not gelatinized.

DETD The term "gelatinization temperatures" is well-understood in the art. Generally the term encompasses temperatures wherein **starch** granules become porous or gelatinize.

DETD The term "internalized proteins" is meant to encompass granule -associated proteins that are not accessible to proteolytic attack unless the **starch** granules are made porous or gelatinized at a gelatinization temperature.

DETD The term "surface-associated proteins" is meant to encompass proteins localized at the surface of the **starch** granules which are non-covalently or covalently bound to the surface of the **starch** granule.

DETD The term "zeins" encompasses surface-associated proteins present on the **starch** granules of **maize** or zeins localized in protein bodies from **maize**, having a molecular weight ranging from about 10 to about 30 kDa as measured by SDS-PAGE.

DETD The term "substantially free of surface-associated proteins" as applied to purified **starch** granules is meant to encompass the removal of 90 to 100% of surface-associated proteins from **starch** granules.

DETD . . . than other comparable preparations, i.e. cosmetics, lotion, foods, etc. As applied to the present invention it is meant to encompass **starch** granules that as a result of protein removal by the method of the present invention are less likely to cause an allergic reaction than **starch** granules wherein the protein is not removed.

DETD Sources of **Starch**

DETD Any suitable **starch**-bearing crop as exemplified above in the present invention can be employed in the processes according to the invention. Simply by way of example, kernels of **maize** (Zea **maize**, inbred line B73) were collected from ears of greenhouse-grown plants at 18-21 DAP, frozen in liquid N.sub.2, and stored at -80.degree. C. Industrial wet-milled **starch** inbred line W64 suspended in steeping solution was provided by Cerestar (Hammond, Ind.). Unless otherwise indicated, steeping solution was removed. . . . washing, and washed granules were air dried. Several examples (FIGS. 1, 3 and 5) utilized laboratory isolated granules prepared from **maize** cultivar B73 as described (Mu-Forster et al. 1996). A polyclonal antibody recognizing **maize** .delta.-zein (10 kDa) was a generous gift from Dr. Joachim Messing (Rutgers University, New Brunswick, N.J.). Thermolysin (protease type X. . .

DETD **Starch** Granule and Amyloplast Isolation

DETD **Starch** granules were isolated by low-speed centrifugation as described (Mu et al. 1994; Mu-Forster et al. 1996). Amyloplasts were isolated from. . .

DETD Protease Digestion of **Starch** Granules

DETD Unless otherwise indicated, proteolytic digestion mixtures contained 50 mg (dry wt.) of isolated **starch** granules, 100 .mu.g of thermolysin and 5 mM CaCl.sub.2 in a volume of 1 ml. Unless otherwise indicated, hydrolysis was. . . as specified in each experiment, and reactions were terminated by addition of EDTA to 20 mM (Xu and Chitnis, 1995). **Starch** granules were centrifuged at 13,000.times.g for 5 minutes. Residual thermolysin was removed by five successive washings with water. Proteins were. . .

DETD Thermolysin digestion of proteins following their release from **starch** granules was conducted as follows: **Starch** granules (50 mg dry wt.) were boiled for 15 minutes in 1 ml of SDS-PAGE sample buffer. The solubilized protein. . .

DETD Granule-associated proteins were recovered by extracting **starch** granules with SDS-PAGE sample buffer (20 .mu.l of buffer per mg dry wt. of granule). Mixtures were then boiled for. . .

DETD Nitrogen content of the **starch** was determined using the improved Kjeldahl method (Method 46-11-A, AACC 1995). Protein content of **starch** granules was obtained by multiplying the percentage of nitrogen content by 5.7 (Tkachuk, 1969).

DETD **Starch** Thermal and Pasting Properties

DETD For differential scanning calorimetry (DSC), dried wet-milled **starch** (4.8 .+-.0.1 mg) was weighed directly into tared aluminum DSC pans. Water was added to a **starch**-water ratio of 1:2, and total sample weights were determined after the pans were sealed. Samples were heated from 30.degree. C.. . . H values were calculated from peak areas and expressed as joules per g of dry matter. Pasting behavior of the **starch** samples was determined using a Rapid Visco-Analyzer (Newport Scientific, Narrabeen, Australia).

DETD Thermolysin Treatment of **Starch** Granules

DETD . . . treatment (FIG. 1, lanes 3 and 5). Finally, to demonstrate that internalized granule polypeptides are thermolysin-sensitive following their removal from **starch** granules, a third set of sample was gelatinized in 2% SDS, and released internalized proteins were then treated with thermolysin. . .

DETD . . . minutes to overnight, a series of lower molecular weight proteins (27, 22 and 10 kDa) are preferentially removed from the **starch** granule (FIG. 1, lane 5 vs. 4). In contrast, internalized proteins such as the 85-kDa SBEII (p85), the 76-kDa SSI. . . waxy protein (p60) are resistant to thermolysin digestion (Mu-Forster et al. 1996). Upon addition of thermolysin following gelatinization of the **starch** matrix, each of these proteins was completely digested

(FIG. 1, lane 1). This indicates that these internalized proteins are thermolysin-sensitive once they are removed from the **starch** granule matrix.

DETD . . . The protein content of 0.13% to 0.14% achieved after thermolysin digestion is about half the levels measured in commercial wet-milled **corn starch** (0.3%) (Hoseney, 1994). This residual protein consists of intrinsically bound granule proteins which remain inaccessible to proteolytic digestion.

DETD Table 1. Protein Content of Untreated and Deproteinized **Maize** Starches.

DETD Wet-milled **starch** granules were incubated with thermolysin at concentration of 0.4 .mu.g mg.sup.-1 in 5 mM CaCl.sub.2. Hydrolysis was conducted at 64.degree. C. or 50.degree. C. for 4 hours. **Starch** granules were washed five times with water to remove residual thermolysin and then air-dried. Control **starch** were treated parallel to deproteinized **starch** at each temperature with thermolysin omitted. Protein concentration of **starch** granule is expressed in percentage of weight of **starch**.

DETD . . . their entirety. However, consistent with a previous study (Mu-Forster et al. 1996), proteins of larger molecular mass such as p85 (**starch** branching enzyme II), p76 (**starch** synthase I) and p60 (waxy protein) and p30 were not removed. SDS-PAGE profiles of proteins in the 10-30 kDa range. . . obtained with antibodies recognizing the .gamma.-zein (16 or 27 kDa) (data not shown). These results clearly establish that digestion of **starch** granules with thermolysin selectively hydrolyzes zein proteins.

DETD There are two possible ways that zeins may associate with **starch** granules. First, if the zeins are located outside of the amyloplast, the association of zeins with **starch** granules would result from interactions of protein bodies with **starch** granules during kernel disruption. Alternatively, under normal growth conditions, zeins could be physically associated with **starch** granules within the amyloplast.

DETD . . . is correct, then isolated amyloplasts should contain very little zein. To test this hypothesis, amyloplasts were purified from 13 DAP **maize** using a gentle mechanical release method and **starch** granules were then isolated from the amyloplasts. As a control, **starch** granules were also isolated by grinding the 13 DAP endosperm in buffer B using a mortar and pestle followed by low speed centrifugation and aqueous washes. Immunoblots probed using the 10-kDa zein antibody clearly show that **starch** granules from purified amyloplasts contained significantly less 10-kDa zein relative to **starch** granule proteins isolated from the 13 DAP **maize** endosperm (FIG. 3, lanes 1 vs. 2). This result demonstrates that in undisrupted kernels, the bulk of the 10-kDa zein is located outside of the amyloplast. The association of the zeins with the **starch** granules must therefore originate from protein bodies which are disrupted under the harsh conditions of kernel grinding and homogenization. The low level of zein associated with the amyloplast-derived **starch** is most likely due to a combination of binding that occurs during the amyloplast isolation procedure, and zein which associate. . .

DETD . . . larger than 30 kDa are unaffected by protease digestion unless granules are pre-gelatinized. Nevertheless, all the proteins extracted from the **starch** granule are intrinsically thermolysin-sensitive, since they are totally hydrolyzed by thermolysin after SDS extraction (FIG. 7, lane 7) (Mu-Forster et. . .

DETD The effect of pH on zein removal is shown in FIG. 5. In this experiment, the pH of wet-milled **starch** granules suspended in steeping solution was adjusted to values between 2 and 11, and samples were subjected to proteolysis. A. . .

DETD . . . Ca.sup.2+ (Feder et al 1971; Tajima et al. 1976), the effects

of Ca.sup.2+ on deproteinization were investigated. With laboratory prepared **starch**, thermolysin failed to exert its proteolytic effect in the absence of exogenous Ca.sup.2+ (FIG. 6, lane 2). When Ca.sup.2+ was. . .

DETD On the other hand, **starch** granules prepared by the industrial wet milling process did not require addition of exogenous Ca.sup.2+. Full surface deproteinization occurred even. . . probably due to use of hard water, which provides sufficient levels of Ca.sup.2+ to activate thermolysin. To demonstrate this point, **starch** granules were washed with 200 mM EDTA to chelate divalent cations prior to incubation with thermolysin. Zein hydrolysis then became. . .

DETD **Starch** Functionality

DETD Table 2 shows the effect of deproteinization on pooled color values of dry **starch** granules. Significantly lower "b" values, which indicate degree of yellowness (27.0% reduction) were observed in the deproteinized **starch** samples. Thermolysin-catalyzed zein removal also resulted in lower H(.degree.).sub.ab values which indicates the degree of hue (31% reduction). However, no significant changes in "a" (redness) and "L" (lightness) values were observed. These results indicate that **starch** granule deproteinization significantly enhances the color of **starch** preparations, which become significantly whiter in appearance. Conversion to a less yellow hue (decreased "b" and H(.degree.)ab values) as the result of **starch** granule deproteinization may due to the fact that pigments which are non-covalently adsorbed to zeins during kernel disruption and steeping. . .

DETD Table 2. Color Profiles of Untreated and Deproteinized **Maize** Starches.

DETD Wet-milled **starch** granules were incubated with thermolysin at concentration of 0.4 .mu.g mg-.sup.1 in 5 mM CaCl.sub.2. Hydrolysis was conducted at 64.degree. C. for 4 hours **Starch** granules were washed five times with water to remove residual thermolysin and then air-dried. Control **starch** were treated parallel to deproteinized **starch** with thermolysin omitted.

DETD

Color Values

	Control **Starch**	Deproteinized **Starch**
L	97.82 .+-. 0.03	98.34 .+-. 0.06
a	-0.51 .+-. 0.02	-0.54 .+-. 0.02
b	2.26 .+-. 0.01	1.65 .+-. 0.01
H(.degree.).sub.ab	4.43 .+-.. . .	

DETD **Starch** Thermal and Pasting Properties.

DETD To determine the effect of zein removal on **starch** pasting properties, RVA analysis was conducted (FIG. 8). **Starch** granules were digested with thermolysin at 64.degree. C. or 50.degree. C. Samples incubated without thermolysin treatment served as controls. At 64.degree. C., no difference was observed between treated samples and controls. At 50.degree. C., deproteinized **starch** granules generated slightly higer cold paste viscosity than controls during the holding period after cooling relative to the parallel treated. . . cold paste viscosities. The minimal effects of deproteinization on thermal and pasting properties demonstrates that the removal of zeins from **starch** granule surfaces by thermolysin does not alter **starch** granule integrity.

DETD To establish whether other protease preparations were as effective as thermolysin in removing zeins from the surface of **corn**

starch granules, a comparative study was undertaken wherein **maize starch** granules were treated with thermolysin or cell extracts of L. Lactis. These cell extracts were described in U.S. Pat. No. 5,246,718 (Haring et al. 1993) as having peptidase activity toward oligopeptides in **starch**. Cells of L. lactis were grown in Lactobacillus MRS both at 37.degree. overnight. Cells were harvested by centrifugation at 5,000.times.g. . .

DETD Enzymatic **starch** granule deproteinization was conducted in a 1 ml suspension containing 0.003 units of peptidase per 50 mg of **starch**, which falls within the suggested range of use. The reaction was carried out at 50.degree. C. for 4 hours with or without calcium present, and was stopped by washing the **starch** granules five items with water. Equivalent samples were treated with thermolysin in parallel. Granule-associated proteins were then extracted and fractionated. . .

DETD As shown in the previous examples, thermolysin treatment removed all of the lower molecular weight proteins from **starch** granules (FIG. 10A, lane 2). However, peptidase activity in the L. lactis cell extract had virtually no effect on zein. . . 10A, lanes 3 and 4). Furthermore, large amounts of soluble protein from the L. lactis cell extract bound to the **starch** granules and could not be removed by the five aqueous washings (FIG. 10A, lanes 3 and 4). Western blotting using the 10-kDa .delta.-zein antibody shows that thermolysin digestion completely removes .delta.-zein from **starch** granules (FIG. 10B, lane 2). However the L. lactis extract was not able to deplete **starch** granules of zein proteins (FIG. 10B, lanes 3, 4 vs. 1).

DETD These results indicate that thermolysin treatment removes zeins from **maize starch** granules by a simple digestion step followed by aqueous washes. While Haring's method might effectively digest oligopeptides from **potato starch** granules using a L. Lactis cell extract in conjunction with physical purification steps (e.g. gas stripping or solvent extraction), it completely lacks the ability to hydrolyze larger proteins such as zeins from **starch** granules of **maize**.

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CLM What is claimed is:

1. A method for purifying **starch** granules obtained from **maize**, comprising treating said **starch** granules with thermolysin at a sub-gelatinization temperature to selectively remove surface-associated proteins, including zein proteins from the surface of the **starch** granules.

3. A method of removing internalized proteins from **starch** granules obtained from **maize**, comprising treating the **starch** granules with thermolysin at a sufficient gelatinization temperature to remove the internalized protein from said **starch** granules.

4. The method according to claim 1, wherein the treatment of the **starch** granules with thermolysin is conducted at a pH of from about 2 to about 11.

5. The method according to claim 1, wherein the surface-associated proteins removed from the surface of the **starch** granules have a molecular weight of about 10 to about 30 kDa as measured by SDS-PAGE.

6. The method according to claim 1, wherein treatment of the **starch** granules with thermolysin is performed in a mixture containing calcium in a concentration of from about 0.5 mM to about. .
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7. Purified **starch** granules obtained from **maize**, which have been treated with thermolysin and which are substantially free of surface-associated proteins otherwise found on the **starch** granule.

8. The purified **starch** granules according to claim 7 having the following characteristics: a) being hypoallergenic relative to **starch** not treated with thermolysin: and b) having an improved flavor relative to **starch** not treated with thermolysin.

9. The purified **starch** granules according to claim 7, characterized as having reduced pigmentation relative to **starch** granules not treated with thermolysin.

10. The purified **starch** granules according to claim 7, wherein the **starch** granules have a protein content of from about 0.13 to about 0.14% relative to the protein content of 0.4 to 1.0% for **starch** not treated with thermolysin.

11. A **starch** product obtained from the process of claim 1.

12. A method of reducing pigmentation of **starch** from **maize** comprising treating **maize** during the steeping or post- steeping processes or isolated **starch** granules with thermolysin at a sub-gelatinization temperature to selectively remove surface-associated proteins from the surface of the **starch** granules.

FILE 'CAPLUS, BABS, CBNB, CEN, CIN, DKILIT, IFIPAT, JICST-EPLUS, PASCAL, PLASNEWS, PROMT, RAPRA, SCISEARCH, TEXTILETECH, USPATFULL, USPAT2, WPIDS, WTEXTILES' ENTERED AT 13:28:13 ON 30 JUL 2002

L1	372062	S	STARCH
L2	43900	S	L1 AND POTATO
L3	1028	S	L2 AND CASSAVA
L4	576	S	L3 AND RICE
L5	296	S	L4 AND BARLEY
L6	171	S	L5 AND MAIZE
L7	109	S	L6 AND CORN
L8	109	S	L7 AND WHEAT
L9	1	S	L8 AND VISCOAMYLOGRAPH